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(21) International Application Number: PCT/SE98/02012 (22) International Filing Date: 6 November 1998 (06.11.98) (30) Priority Data: 9704076-0 6 November 1997 (06.11.97) SE (71) Applicant (for all designated States except US): A+ SCIENCE INVEST AB [SE/SE]; P.O. Box 3096, S-400 10 Göteborg (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): ORWAR, Owe [SE/SE]; Haga Kyrkogata 4, S-411 23 Göteborg (SE). LUNDQVIST, Anders [SE/SE]; Fjärde Långgatan 28, S-413 27 Göteborg (SE). ERIKSSON, Peter [SE/SE]; Dr. Saléns Gata 10, S-413 22 Göteborg (SE). CHIU, Daniel [US/US]; 36900 Papaya Street, Newark, CA 94560 (US). (74) Agent: AWAPATENT AB; P.O. Box 11394, S-404 28 Göteborg (SE).		(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHOD FOR ELECTRO-PERMEABILISATION OF INDIVIDUAL CELLULAR AND ORGANELLAR STRUCTURES AND USE THEREOF		
(57) Abstract <p>The invention relates to a method for permeabilisation of a cell structure consisting of a single cell, an intracellular structure or an organelle comprising the following steps: (a) microelectrodes, preferably two carbon fibre electrodes, are provided, (b) the microelectrodes are connected to a power supply, (c) the electrodes, individually controlled by high-graduation micromanipulators, are placed close to the cell structure at an appropriate inter-electrode distance, and (d) a highly focused electric field of a strength sufficient to obtain electroporation is applied between the electrodes. The method may be used in order to transfer cell impermeant solutes, such as drugs or genes, into the cell structure or out of the cell structure, in biosensors, in the treatment of tumours and neurodegenerative diseases and in the study of biophysical processes.</p> <div data-bbox="974 1144 1437 1963"> </div>		

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METHOD FOR ELECTRO-PERMEABILISATION OF INDIVIDUAL
CELLULAR AND ORGANELLAR STRUCTURES AND USE THEREOF

Field of the invention

The present invention relates to a highly spatially resolved method for permeabilisation of cell structures, organelle structures and cell-like structures in order to transfer compounds into or out of the structures. It also relates to use of this method.

Background of the invention

During the last two decades there has been a tremendous growth in experimental methods that allow for biochemical and biophysical investigations of single cells. Such methods include patch clamp recordings which can be used for measurement of transmembrane currents through a single ion channel (O. P. Hamill, A. Marty, E. Neher, B. Sakman, F. J. Sigworth, *Pflegers Arch.* **391**, 85-100 (1981)); laser confocal microscopy imaging techniques that can be used to localise bioactive components in single cells and single organelles (S. Maiti, J. B. Shear, R. M. Williams, W. R. Zipfel, W. W. Webb, *Science*, **275**, 530-532 (1997)); use of near field optical probes for pH measurements in the cell interior; and use of ultra-microelectrodes for measurement of release of single catechol- and indol-amine-containing vesicles (R. H. Chow, L. von Ruden, E. Neher, *Nature*, **356**, 60-63 (1992) and R. M. Wightman, J. A. Jankowski, R. T. Kennedy, K. T. Kawagoe, T. J. Schroeder, D. J. Leszczyszyn, J. A. Near, E. J. Diliberto Jr., O. H. Viveros, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10754-10758 (1991)).

Although numerous high-resolution techniques exist to detect, image and analyse the contents of single cells and subcellular organelles, few methods exist to control and manipulate the biochemical nature of these compartments. Most compounds for biological and medical use that are of interest to include in cells are polar. Polar solutes are

cell-impermeant and unable to pass biological membranes unless specific transporters exist. Often in experimental biology as well as in biochemical and clinical work, polar solutes need to be administered to the cytoplasm of cells or to the interior of organelles. Examples of such polar solutes are nanoparticles, dyes, drugs, DNAs, RNAs, proteins, peptides, and amino acids. At present, it is extremely difficult, for example, to label a cell in a cell culture with a dye, or transfect it with a gene without labelling or transfecting its adjacent neighbour. It is even more difficult to introduce polar molecules into organelles because of their size which many times is smaller than the resolution limit of a light microscope, or at least less than a few micrometers in diameter.

Microinjection techniques for single cells and single nuclei have also been described (see e.g. M. R. Capecchi, *Cell*, **22**, 479-488 (1980), but these get increasingly difficult to implement as the size of the cell or organelle decreases. For cells and organelles measuring only a few micrometers in diameter or less, microinjection techniques become virtually impossible to use.

It has for a long time been recognised that cell membranes can be permeabilised by pulsed electric fields (see e.g. Zimmermann, U. *Biochim. Biophys Acta*, **694**, 227-277 (1982); Tsong, T. Y. *Biophys. J.*, **60**, 297-306 (1991); Weaver, J. C. *J. Cell. Biochem.*, **51**, 426-435 (1993)). This technique is called electroporation. The membrane voltage, V_m , at different loci on phospholipid bilayer spheres during exposure in a homogenous electric field of duration t , can be calculated from:

$$V_m = 1.5 r_c E \cos \alpha [1 - \exp(-\tau/t)] \quad (1)$$

where E is the electric field strength, r_c is the cell radius, α , the angle in relation to the direction of the electric field, and τ the capacitive-resistive time constant. Pore-formation will result at spherical coordi-

nates exposed to a maximal potential shift, which is at the poles facing the electrodes ($\cos\alpha=1$ for $\alpha=0$; $\cos\alpha=-1$ for $\alpha=\pi$). Generally, electric field strengths on the order of from 1 to 1.5 kV/cm for durations of a few μ s to
5 a few ms are sufficient to cause transient permeabilisation in 10- μ m-outer diameter spherical cells. A recent study shows that isolated mitochondria, because of their correspondingly smaller size, require 7-10-fold higher electric field strengths to incorporate a 7.2-kilobase
10 plasmid DNA (J-M. Collombet, V.C. Wheeler, F. Vogel, & C. Coutelle *J. Biol. Chem.*, 272, 5342-5347 (1997)). Mitochondrial outer-membrane fusion at lower electric field strengths of about 2.5 kV/cm has also been observed.

Traditionally, electroporation is made in a batch
15 mode allowing for administration of polar solutes into several millions of cells simultaneously. The electrodes producing such fields can be several square centimetres and the distance between the electrodes several centimetres, thus requiring high-voltage power sources to obtain
20 the needed electrical field strength to cause electrically induced permeabilisation of biological membranes.

One advantage of electroporation compared to micro-injection techniques is that electroporation can be extremely fast, and precisely timed (see e.g. K. Kinoshita,
25 K., Jr., I. Ashikawa, N. Saita, H. Yoshimura, H. Itoh, K. Nagayama, & A. Ikegami *J. Biophys.*, **53**, 1015-1019 (1988); M. Hibino, M. Shigemori, H. Itoh, K. Nagayama, & K. Kinoshita, K., Jr., *Biophys. J.*, **59**, 209-220 (1991)) which is of importance in studying fast reaction phenomena.

30 Instrumentation that can be used for electroporation of a small number of cells in suspension (K. Kinoshita, Jr., & T. Y. Tsong, *T. Biochim. Biophys. Acta*, **554**, 479-497 (1979); D.C Chang, *J. Biophys.*, **56**, 641-652 (1989; P. E. Marszalek, B. Farrel, P. Verdugo, & J. M. Fernandez, *Biophys. J.*, **73**, 1160-1168 (1997)) and for a small number
35 of adherent cells grown on a substratum (Q. A. Zheng, & D.C. Chang, *Biochim. Biophys. Acta*, **1088**, 104-110 (1991);

M. N. Teruel, & T. Meyer *Biophys. J.*, **73**, 1785-1796 (1997)) have also been described. The design of the electroporation device constructed by Marszalek et al. is based on 6 mm long 80 μm diameter platinum wires that are glued in a parallel arrangement at a fixed distance of 100 μm to a single glass micropipette. The design by Kinoshita and Tsong uses fixed brass electrodes spaced with a gap distance of 2 mm, the microporator design of Teruel and Meyer relies on two platinum electrodes that are spaced with a gap distance of about 5 mm, and the electroporation chamber design by Chang uses approximately 1 mm-long platinum wires spaced at a distance of 0.4 mm. It is obvious, that these electroporation devices create electric fields that are several orders of magnitude larger than the size of a single cell which typically is 10 μm in diameter, and thus can not be used for exclusive electroporation of a single cell or a single organelle or for electroporation inside a single cell. Electroporation devices for clinical applications has also been designed. Examples include devices for electroporation-mediated delivery of drugs and genes to tumours (WO 96/39226) and to blood cells (US Pat. 5,501,662). Likewise, these can not be used to create a highly localised electric field for electroporation of a single cell, a single organelle, or a population of organelles within a cell.

Summary of the invention

One of the major disadvantages with the known techniques is that they are not applicable for permeabilisation of single cells or single intracellular organelles.

The present invention provides a highly spatially resolved technique to alter the biochemical content of single cells and organelles, based on permeabilisation of phospholipid bilayer membranes by pulsed electric fields, i.e. so called electroporation.

An advantage the method according to the present invention compared to known methods for electroporation is

that the method according to the invention is characterised by an extremely high spatial resolution defined by highly focused permeabilising electric fields. Such highly focused electric fields are obtained by using a pair of electroporation electrodes with outer diameters in the range of a few manometres to a few micrometers. This enables electroporation of single cells and even intracellular structures. The electrodes are controlled individually with high-graduation micropositioners, thereby enabling precise electrode alignment with respect to a structure to be permeabilised. During the effective pore-open time, cell-impermeant solutes added to the extracellular or extraorganellar medium can enter the cell or organelle interior by diffusion.

In contrast to the known microinjection techniques for single cells and single nuclei, the present invention can be applied for biological containers of subfemtoliter ($< 10^{-15}$ l) volumes or less than a few micrometers in diameter, which is another important advantage of the invention.

Furthermore, the present invention distinguishes itself from prior art in that electroporation with single-cell or subcellular spatial resolution is accomplished by applying the electric field through nanometer- and micrometer-diameter electrodes with extremely short inter-electrode distances. The electrodes are controlled individually by high-graduation micromanipulators, allowing precise focusing of the electric field between the electrodes. Electroporation of individual cells and individual organelles can thereby be accomplished. Electroporation can with the present invention be performed in such a way that only a target cell is permeabilised and not its adjacent neighbour. Also, individual cellular processes can be electroporated. Even spatially well-defined intracellular domains with a targeted class of organelles can be held under a localised electric field with this invention, thereby enabling transfer of polar solutes

into organelles. Applications of electroporation of organelles include alterations of the mitochondrial genome. It is well known that mutations in the mitochondrial genome can lead to a multitude of diseases, and that gene therapy can potentially be of major importance. So far, however, mitochondria has to be isolated from the cells before transfer of the new gene fragment into the mitochondria can be performed. Then, the mitochondria has to be reinserted into the cell. The technique according to the invention makes it possible to directly insert genes into the mitochondria when they are contained inside a cell. This is a significant advancement over traditional schemes for transfection of mitochondria.

In addition to the high spatial resolution achieved by using nano- and micro-electrodes, the technique according to the invention avoids the use of expensive high-voltage pulse generators, and complicated microchamber mounts. The method according to the invention can in principle be battery-operated because the spacing between the electrodes is small, typically 20 μm or less, which result in a high electric field strength with a small amplitude voltage pulse. This technique is the first demonstration of selective solute-transfer into biological structures using highly focused electric fields of single-cell and subcellular dimensions.

The method according to the invention can be additionally used for biosensor applications where a cell or a cell-like structure is placed in a permeabilising dc or ac electric field while supplemented with drugs or other compounds of interest. A special application is the combination of electroporation and miniaturised chemical separations, where hollow liquid electrodes made of fused silica or similar materials are used.

With ultramicroelectrodes, such as carbon fibre electrodes, controlled by high-graduation micromanipulators, used according to the present invention, it is easy

to focus the electrical field to very well-defined regions.

Thus, the present invention relates to a method for permeabilisation of a cell structure consisting of a
5 single cell or a cell-like structure, an intracellular structure or an organelle characterised in that it comprises the following steps:

- (a) microelectrodes are provided;
- (b) the microelectrodes are connected to a power
10 supply;
- (c) the electrodes, individually controlled by high-graduation micromanipulators, are placed close to the cell or organelle structure or inside a single cell at an appropriate inter-electrode distance using high-
15 graduation micromanipulators;
- (e) a highly focused electric field of a strength sufficient to obtain electroporation is applied between the electrodes.

The cell structure can be any kind of cell or cell-
20 like structure, such as a cell in a primary cell culture, a cell in a tissue slice or a tissue, an in vivo cell, a liposome, or an intracellular cell structure, such as an organelle,.

The method according to the invention may be used
25 either for transferring solutes from an extracellular medium into a permeabilised cell structure, or for transferring solutes entrapped in the cell structure out to the extracellular medium. The method according to the invention may also be used for transferring a substance
30 into or out from an organelle, even when the organelle is located inside a cell.

The method is well-suited for the study of cellular migration, proliferation, growth, and differentiation, as well as a multitude of biochemical and biophysical
35 events. It also opens up new possibilities for highly spatially resolved distribution of nanoparticles, drugs, genes and different biochemical markers, such as dyes

into single cells or organelles both isolated and *in situ*. The method may be useful in clinical applications as a vehicle to administer drugs and genes to patients.

5 The method may also be useful for biosensor applications. In particular, a single cell can be placed in a permeabilising ac or dc field, thereby allowing cell-impermeant molecules that affect intracellular chemistry, including activation of receptors present on the surface of various organelles to be activated. In this way a compound library that acts on intracellular chemistry can be
10 screened for biological activity. The compounds of interest can then be added to the cell solution using a perfusion system or a syringe. In a special case the compounds of interest can be delivered by a fused silica electrophoresis capillary of narrow inner dimensions. Because
15 the electrophoresis capillary is connected to a voltage source, the electrophoresis capillary can be viewed as a liquid-filled electrode. If the outlet end of the electrophoresis capillary is placed close enough to the cell membrane, and an electric field strength sufficient to
20 cause dielectric membrane breakdown is applied, compounds injected into the capillary will cross the cell-membrane barrier and enter the cell interior where they can act on intracellular receptors and intracellular chemistry. Because
25 electrophoresis is a chemical separation technique, it can be used as a fractionation and screening method for biologically important compounds.

30 The characterising features of the invention will be evident from the following description and the appended claims.

Brief description of the drawings

In the description and the examples below reference is made to the accompanying drawings on which:

35 Fig. 1 shows a preferred embodiment of an apparatus used for carrying out the method according to the invention.

Fig. 2 A - C are schematic drawings of the positioning of the electrodes and the electrical field for electroporation of a single cell. Fig. 2 A illustrates the situation before electroporation, when all solutes are present in the extracellular volume. Fig. 2 B illustrates the situation during application of a voltage pulse when a highly focused electrical field is created over the cell resulting in permeabilisation and diffusion of the solutes into the cell. Fig. 2 C illustrates the situation after the electroporation when the membrane pores are resealed and the solutes are trapped inside the cell. It is also feasible at high electric field strengths to electroporate intracellular organelles using this scheme. This results in pore-formation both in the cell membrane and in the organelle structures.

Fig. 3 shows a set-up that can be used for electroporation of intracellular organelles. Fig. 3A shows a pair of electrodes inserted into a cell. The electrode tips are placed so that an organelle to be permeabilised is located between the electrode tips. Fig. 3B shows application of an electric field of a strength sufficient to cause pore-formation in the organelles. Organelle-impermeant molecules injected to the cell can thereby diffuse into the organelle. In fig. 3C the electrodes are withdrawn, excess molecules are removed from the cytoplasm, and the molecules are exclusively located inside a population of permeabilised organelles.

Fig. 4 A and B show electroporation of cells using electrolyte-filled electrodes. Fig. 4A shows a liquid-filled fused silica electrode with its outlet placed close to the membrane of a single cell. The electrolyte inside the capillary electrode contains cell-impermeant molecules. Fig. 4 A il-

illustrates the situation when a permeabilising field is applied and the molecules contained in the electrolyte migrate by electroosmosis and electrophoresis, to the outlet end of the capillary electrode.

Fig. 5 illustrates an example of a clinical application of the method according to the invention. Two electrodes are placed in a cellular structure in human brain. The positioning of the electrodes can be performed using a stereotactic atlas, represented in the figure by the Cartesian coordinate system, and stereotactic micropositioners. One electrode is hollow for perfusion of e.g. drugs or genes. Perfusion can be achieved as shown here with a syringe or by some other means, including electrophoresis.

Fig. 6 A and B show video images taken through a microscope demonstrating electroporation of progenitor cells to incorporate fluorescein. Fig. 6 A is a brightfield image of two progenitor cells. The cell on the top was electroporated in the presence of fluorescein. Fig. 6 B is a fluorescence image demonstrating that the fluorescence is uniquely localised to the upper cell with virtually zero spillover of fluorescein to the neighbouring cell.

Fig. 7 shows photomicrographs demonstrating differential fluorescence staining resulting from electroporation of fluorescein into single progenitor cells at high, and low electric field strengths, respectively. Fig. 7 A shows two cells electroporated at plasma membrane superthreshold potentials of approximately 2 V (ten pulses at 0.5 Hz repetition rate), both of which display a punctuate cytoplasmic fluorescence pattern due to incorporation of dye into organelles. Fig. 7 B shows images of three cells after electroporation

at the plasma membrane threshold potential of about 1 V (ten pulses at 0.5 Hz repetition rate) where the fluorescence is diffuse and evenly distributed over the entire cell, demonstrating electroporation of fluorescein into the cytoplasm of the cells.

Detailed description of the invention

With the method according to the present invention it is possible to permeabilise cell structures or cell-like structures and thereby to transfer polar, cell-impermeant solutes, such as nanoparticles, drugs, dyes, RNA, DNA, proteins, and peptides, into or out of cell-like structures. The technique is based on the use of a highly focused electric field that is applied over the cell structure by means of microelectrodes placed close to the cell structure and a voltage source. The microelectrodes are controlled by micromanipulators, preferably high-graduation micromanipulators, and can be translated in three-dimensional space in increments of tens to a few hundreds of nanometres. Preferably two microelectrodes are used, these can be made of carbon fibre, metal or other electrically conductive material. The electrodes can be hollow for administration of, for example, drugs, genes, or dyes through the inner channel. The electrical current needed for electroporation of a cell structure can also be carried by an electrolyte contained in the inner channel. Such hollow capillary electrodes can be made of fused silica or similar materials, thereby enabling delivery of the drug, gene, or dye to the cell or organelle through electrophoresis or electroosmosis. The diameter of the electrodes are preferably only a few nanometres to a few micrometers in diameter. In the case of hollow electrodes, the inner channel diameter can be a few nanometres to a few tens of micrometers in diameter, whereas the outer diameter can be a few micrometers to several hundred micrometers. Preferably, two microelec-

trodes are used. However, a single microelectrode for electroporation can be used together with a mesoscopic or larger electrode held at ground or at negative potential. The electrodes can be placed adjacent to a cell for electroporation of the cell membrane and organelles contained inside the cell or the electrodes can be placed inside the cell for exclusive electroporation of the organelles contained inside the cell.

The voltage source generates a voltage pulse, with a waveform that can be square, exponential, or of any other form. It is also possible to use both DC currents and AC currents.

The electrical fields needed to cause electroporation varies largely depending on the type and the size of the treated cell structure. The appropriate field strength can be calculated by man skilled in the art by using see equation 1 given in the background description of this application. The electric field strength is adjusted by the voltage and the inter-electrode distance. Appropriate field strengths may be in the range of from 0,01 kV/cm to 100 kV/cm. Appropriate inter-electrode distances are distances smaller than 10 mm, preferably smaller than 100 μm . If a single mammalian cell is to be treated it may be appropriate to use an inter-electrode distance of from 0,1 μm to 30 μm , and a voltage of from 100 mV to 100 V.

The duration of the voltage pulse may vary from a few microseconds to several minutes, also depending on the type and the size of the treated cell structure. The length of the voltage pulse is preferably from 1 μs to 500 ms.

During application of the voltage pulse, the cell structure is permeabilised through pore formation, allowing polar solutes which otherwise can not pass biological bilayer membranes, to enter or escape the interior of the cell structure through diffusion. The spatial resolution of the method according to the invention is dictated by

the size of the electrodes, which can be made to be only a few nanometres in diameter, and the gap distance between the electrodes, which can be made to be a few nanometres to a few micrometers, thereby allowing for electroporation even of the smallest of intracellular organelles and e.g. nanobacteria.

When the method according to the invention is used for transferring biological markers, nanoparticles, dyes, and other particles into cell structures some of the applications are the study of cellular migration, proliferation, growth, differentiation, as well as other biochemical and biophysical events. The method might prove useful in clinical applications as a vehicle to administer drugs and genes to patients. For clinical applications, in particular, a hollow electrode configuration is suitable in which the compound to be electroporated into the cell is administered through a small channel in the electrode.

When the method according to the invention is performed *in vitro* in order to transfer solutes into a cell structure, such as a tissue slice, a primary culture, a cell line or a preparation of an organelle, an appropriate apparatus may be the preferred embodiment illustrated in figure 1, comprising two carbon fibre microelectrodes 1, 2, with outer diameters in the nanometre-to-micrometer size range, connected to a voltage generator 3. Preferably, the electrodes are connected to the voltage generator via vials 4, 5, containing for example 3 M KCl, mercury, or silver glue and silver wires 6, 7. The cell structure 8 is typically held in e.g. a petri dish 9 in some kind of physiological buffer supplemented with the compound to be incorporated into the cell. In order to facilitate viewing and hence positioning of the electrodes in relation to the cell structure it is possible to use a microscope. In the preferred embodiment shown in figure 1 the petri dish is situated on an inverted microscope stage 10, and viewed through a microscope objective

11. The electrodes 1, 2, are, by means of three-dimensional micropositioners 12, 13, preferably of a high graduation type, positioned close to each side of the cell structure, preferably in an opposing linear arrangement so that the electrodes face each other with the cell structure in the middle. This is performed in such a way that the electric field produced between the electrodes is highly focused over the structure to be electroporated.

10 Since the distance between the tips of the electrodes is very short, just a few micrometers, only a low-voltage generator is typically needed to produce the required electrical field strengths for electroporation of biological membranes. Preferably, a rectangular DC voltage pulse is applied over the cell and pores are created in the membrane through which solutes diffuse into the interior down their concentration gradient.

Depending on the composition of the buffer the conditions are changed at the electrodes. Electrochemical reactions at the electrodes, e.g. reduction of water and oxidation of chloride, causes some loss in voltage and the effective voltage should be calculated for every given set of electrode materials and buffer systems.

Figure 2A - C are schematic drawings of the positioning of the electrodes and the electrical field for electroporation of a single cell. Fig. 2 A illustrates the situation before electroporation, when all solutes are present in the extracellular volume. Fig. 2 B illustrates the situation during application of a voltage pulse when a highly focused electrical field is created over the cell resulting in permeabilisation and diffusion of the solutes into the cell. Fig. 2 C illustrates the situation after the electroporation when the membrane pores are resealed and the solutes are trapped inside the cell. It is also feasible at high electric field strengths to electroporate intracellular organelles using

this scheme. This result in pore-formation both in the cell membrane and in the organellar structures.

After electroporation of the cell structure, washing of the cell dish can be performed in order to remove excess solutes if needed. The buffer is then replaced by a
5 solute-deficient buffer or a culture media.

With the present invention it is also possible to exclusively electroporate organelles. The organelles can be contained inside a single cell or they can be isolated
10 from a single cell or a population of cells. As stated above, the strengths of the pulse depend on what structure to be electroporated and on the distance between the electrodes. Small organellar structures, like endoplasmic reticuli, and mitochondria, require a higher voltage to
15 create pores in the membrane. The pulse duration can also be varied in the range of a few microseconds to several minutes depending on membrane structure but also on the structure of the compound to be incorporated. Large molecules with low diffusion rates require longer periods of
20 time to move into the cell. For electroporation of organelles inside a cell, intracellular electroporation electrodes are preferentially used. These electrodes can have electrically insulating shanks, so that the part of the electrode that comes into contact with the cell membrane
25 does not result in electrically-induced pore-formation. The tips of the electrodes can be made of electrically conductive materials, such as deposited metals, or carbon fibre. Alternatively, hollow-fibre electrodes filled with an electrolyte can be used for such purposes. The physical
30 dimensions of intracellular electrodes for electroporation of organelles can range from a few nanometres to a few micrometers in diameter.

A preferred embodiment for electroporation of intracellular organelles is shown in figure 3. The electrodes
35 1, 2 used in this set-up have a sharp tip 14, 15 that is made of a conductive material, and electrically insulated shanks. The tip diameter of such electrodes is between a

few nanometres and a few micrometers in diameter. Fig. 3 A shows a pair of electrodes 1, 2 inserted into a cell surrounded by a cell membrane 16. The insertion and positioning of the electrodes is performed with high-graduation micromanipulators (not shown). The electrode tips 14, 15 are placed at an intracellular domain of interest. The electrodes are arranged so that the organelle 17 to be permeabilised is located between the electrode tips. In Fig. 3 B an electric field of a strength sufficient to cause pore-formation in the organelle 17 has been applied. Organelle-impermeant molecules 18 injected to the cell with a microsyringe, or by some other means, can thereby diffuse into the permeabilised organelle 17. This procedure is repeated until the desired number of organelles have been permeabilised. In fig. 3C the electrodes are withdrawn, excess molecules are removed from the cytoplasm using degradative pathways, or by some other means, and the molecules are exclusively located inside a population of permeabilised organelles.

Another application of the present invention is for use with biosensor techniques. In particular, by applying a permeabilising electric field over a single cell, intracellular chemistry and organelles can be used for biosensing purposes. As an example, inositoltriphosphate, which activates receptors on endoplasmic reticuli, can be assayed for using such schemes. The compound is simply added to the buffer surrounding the permeabilised cell and will diffuse into the cell interior and bind to receptors on endoplasmic reticuli. Upon binding of inositoltriphosphate to the receptors, endoplasmic reticuli will liberate calcium ions. If the cell is then supplemented with a fluorogenic calcium chelating dye, such as fluo-3, the receptor activation can be measured as an increase in fluorescence.

When hollow fibre electrodes are used, according to the present invention, the cell-impermeant molecules added to the intra-electrode solution can be administered

to the cell using a perfusion system, such as a microsyringe pump or a peristaltic pump. According to another scheme, electrolyte-filled fused silica capillaries with narrow inner diameters are used as electroporation electrodes. With these electrodes, the cell-impermeant molecules contained in the electrolyte solution can be delivered to a cell using electrophoresis or electroosmosis as shown in figure 4. The applied electric field causes pore formation in the cell as previously discussed. Because the components inside the electrode electrolyte will be separated based on their charge-to-frictional drag ratio, this method can be used for, for example, biosensor applications. For electroporation of cells it is possible to use either electrodes with conductive tip for application of the electric field necessary to create pore-formation or electrodes filled with an electrolyte that carries the electric current necessary to create pore-formation. In the case of electrodes with an conductive tip, cell-impermeant molecules supplemented to the liquid contained in the hollow electrodes can be administered to the cell using a perfusion system, such as a syringe pump or peristaltic pump. In the case of using hollow electrodes in which the permeabilising current is carried by the electrolyte, the molecules contained in the electrolyte of the hollow electrode can additionally be delivered to the cell structure by electrophoresis or electroosmosis. When using electrophoresis or electroosmosis, the components in the hollow electrode can be separated based on their charge-to-frictional-drag ratio. This opens up possibilities for performing organelle-sensor-based detection of species fractionated by electrophoresis.

Electroporation of cells using liquid-filled fused silica electrodes with tapered tips is illustrated in fig. 4 A and B. Such tapered tips can be created by pulling the fused silica capillary in a flame, etching in hydrofluoric acid, or by grinding.

Fig. 4 A shows an electrolyte-filled fused silica electrode 1 with its tapered outlet placed close to the membrane 16 of a single cell. The electrolyte inside the capillary contains cell-impermeant molecules 19 injected into the capillary hydrodynamically, or by some other means. In this case, the supplemented cell-impermeant molecules 19 activate receptors 20 on organelles 17. When a permeabilising field is applied, by application of positive voltage to the inlet end of the capillary electrode, the molecules contained in the electrolyte will migrate by electroosmosis and electrophoresis, to the outlet end of the capillary electrode. Because the electric field is chosen to be strong enough to permeabilise the cell membrane, the cell-impermeant molecules supplemented to the electrolyte contained in the electrode will pass the pores 21 formed in the cell membrane and can, for example, activate receptors on organelles, as shown in Fig. 4 B. Because the electric field is chosen to be strong enough to permeabilise the cell membrane, the cell-impermeant molecules supplemented to the electrolyte contained in the electrode will pass the pores formed in the cell membrane and can, for example, activate receptors on organelles. Electrodes of similar sort but of smaller dimension can be used for electroporation of organelles.

If the method according to the invention is carried out *in vivo* it is possible to use a surgical microscope, in order to view the cell structure. Furthermore, it is possible to use a stereotactic device for the positioning of the electrodes. When the method is performed *in vivo* it is of course not possible to place the cell structure in some sort of container with buffer. Instead, the compound to be incorporated into the cell structure is administered in a physiological buffer either separately via a catheter or directly via a hollow fibre electrode. When hollow fibre electrodes are used the compound is administered to the cell structure through a small channel in the electrodes which is coupled to a syringe con-

trolled by a micrometer screw or a microinjection pump to enable administration of an exact volume. The possibilities to add the compound either at the same time as the electroporation occurs or with some time delay make this technique very useful. A very high concentration is achieved locally at the selected cell and diffusion into it will be faster due to the greater concentration gradient. The lower consumption of the compound and the minimisation of the problems due to the washing procedure are some other advantages. Many times a focal administration, i.e. administration directly to the malfunctioning set of cells, of drugs or genes can be expected to be far superior than intraperitoneal, oral, intraventricular, or any other kind of commonly employed drug-administration technique. Intracellular drug-and-gene-administration in vivo can be accomplished with the method according to the invention. Because of the extremely small dimensions of the electrodes, in combination with the low voltages applied, very little tissue trauma is expected. Furthermore, the positioning of the electrodes and the subsequent gene or drug delivery is very precise. It has been shown that microdialysis probes, which are on the order of a hundred times larger than the electrodes used in the method according to the present invention, cause very little tissue trauma and disruption of local metabolism.

With the method according to the invention, used in combination with gene therapy, it will be possible to "re-program" cells; either in order to make a malfunctioning cell work in the correct way, or to give a cell a new function.

It is thus possible to use the method according to the invention in therapies for and treatment of different diseases and conditions caused by the malfunction of single cells or a small population of cells, such as Parkinson's disease and brain tumours, which is further illustrated below.

Many diseases, may they be genetically acquired or not, result in metabolic disruptions. For example, Parkinson's disease caused by degeneration of neurons in the Nigro-striatal pathway result in malfunctioning in the biochemical machinery for production of dopamine in an isolated population of cells. This in turn results in motorbehavioral deficits. The standard treatment of Parkinson's disease is by oral administration of L-DOPA, a precursor of dopamine. Alternatively, grafted tissue with neuronal cells producing dopamine is transplanted into the patient's brain. Intracellular drug or gene administration in vivo into the appropriate brain structures can be accomplished using an electroporation procedure similar to that described in the two above examples and used as a therapeutic strategy.

Experimental treatment of brain tumours using genetically engineered viruses for gene delivery has been used with anecdotal reports of success. The use of viruses as a delivery system has limitations in that it might pose a potential hazard if the virus mutate. Using an electroporation procedure for gene delivery, e.g. "suicide genes" for cytokine deaminase or thymidine kinase, similar to that described in the examples below, eliminates the need for the use of virus delivery systems in cancer therapy.

Figure 5 is a schematic illustration of treatment of brain tumours with the method according to the invention. Two electrodes, preferably at least one hollow fibre electrode for perfusion of e.g. drugs or genes, are placed in a cellular structure in human brain. The positioning of the electrodes can be performed using a stereotactic atlas, in the figure represented by the Cartesian co-ordinate system, and stereotactic micropositioners. Perfusion can be achieved as shown in the figure with a syringe or by some other appropriate means. The positioning of the electrodes and the applied electrical

field can be varied so a desired number of cells are electroporated.

The solutes to be electroporated into cells are then simply administered through the narrow channel in the
5 centre of the electrode or electrodes by application of a flow by means of a syringe pump or a peristaltic pump or any other type of solution pumping system including electrophoresis.

An especially interesting possibility is to use battery-operated perfusion/electroporation implants in biotolerable materials for continuous application of solutes into cells. Because such low potentials are required, batteries providing voltages in the range of from a few to about 20 volts can be used. These battery-operated
10 electroporation units can be made small, they can be included on a chip measuring only a few millimetres squared. Such a system could include a solute-containing solution reservoir, two electrodes for electroporation, electronic circuitry for timed delivery and control of
15 the electroporation parameters, i.e. pulse profile, pulse duration, repeat, and amplitude as well as a battery source, and a reservoir refill inlet.

The electrodes used according to the present invention may have any appropriate shape or configuration.
25 They may be of the type illustrated in figure 1. They may also, for example, be one pair or several pairs of rod-like electrodes that penetrate the full length of the structure to be electroporated. When an electric field is applied over these rod-like electrodes all cells exposed
30 will be electroporated. This allows for a higher turn over number and faster treatments.

When the method according to the invention is used for transferring entrapped solutes out of cell structures, so called reversed electroporation, some of the
35 applications are drug delivery to single cells or small populations of cells. This is e.g. used in order to permeabilise cells and cell-like structures, such as

liposomes in order to transfer cell impermeant solutes from their inside to the extracellular media. The concentration gradient for the solutes then have the opposite direction i.e. high solute concentrations inside the cell structure and low solute concentrations outside the cell structure. In particular, liposomes with entrapped drugs can be emptied in a controlled way close to a target cell simply by applying a DC voltage pulse over its membrane through carbon fibre microelectrodes positioned in the same way as described above. The use of such liposome-based delivery of drugs triggered by electroporation can be used in basic research as well as in the pharmaceutical industry, e.g. for compositions with delayed release.

It is also possible to make cells discharge solutes present in their cytoplasm by application of an electric field identical as described above for solute-transfer into cells using electroporation.

The invention will be further illustrated in the examples below, which in no way limit the scope of the invention.

Example 1. Electroporation of progenitor cells from mature rat brain to incorporate a fluorescent marker

Progenitor cells were cultured according to standard procedures (T. D. Palmer, J. Ray, F. H. Gage, *Mol. Cell. Neurosci.*, **6**, 474-486 (1995)) and plated onto number 1, 1-inch circular coverslips coated with poly-D-ornithine and laminin. The cells were allowed to stand overnight to adhere to the glass surface. The cells were incubated in a humid atmosphere at 37°C with 5% CO₂ and 95% air. For the electroporation, the cell dishes were mounted into a circular low-rim polycarbonate holder using mounting grease. The polycarbonate holder with the cover slip was mounted on the stage of an inverted microscope (Leica DM IRB). The cells were held in a HEPES-buffered saline containing 137 mM NaCl, 0.4 mM MgCl₂, 0.64 mM KH₂PO₄, 3.0 mM NaHCO₃, 0.41 mM MgSO₄, 5.4 mM KCl, 20 mM HEPES, 1.26 mM

CaCl₂, and 5.5 mM D-glucose (pH adjusted to 7.4 with NaOH). This HEPES buffer was supplemented with fluorescein (sodium salt, 5 µM), a highly fluorescent charged species.

5 To electroporate the cells, two carbon fibre micro-electrodes (Pro CFE, Axon Instruments, Foster City, CA) connected with the power supply by a liquid junction of KCl (3 M) and a thin silver wire, were placed in the cell bath with a distance of about 15 µm between the tips and
10 with the cell positioned in the centre between the two tips. This configuration is illustrated in figure 3 A. Electroporation of the cell membrane was accomplished by applying one-to-ten pulses of DC voltage yielding membrane potentials of 0.5-to-1 V with a duration of about 1
15 ms over the tips using a low-voltage pulse generator (Digitimer Stimulator, DS9A, UK). It should be noted that different voltages, durations, pulse profiles, and also alternating currents can be employed if needed. During application of the voltage pulse, a highly focused electrical field is created over the cell. The presence of
20 the field results in permeabilisation of the cell through pore formation and the solute molecules diffuses into the cell down their concentration gradient, which is illustrated in figure 3 B. Following application of the electrical pulse the membrane pores are resealed and the an-
25 alytes are then trapped inside the cell. Finally, the cell dishes were washed with HEPES buffer solution. The final situation is illustrated in figure 3C.

The cells were then viewed in the microscope using
30 excitation of the fluorescein at 488 nm using an Ar-ion laser (Spectra Physics model 2025-05, Sunnyvale, CA). The laser light was sent through a 488-line interference filter followed by a spinning disk to break the coherence and scatter the laser light. The laser was collected by a
35 lens and sent through a fluorescein filter cube (Leica I-3) into the objective to excite the fluorophores. The resulting fluorescence was collected by the same objec-

tive and the image was detected by a 3-chip colour CCD-camera (Panasonic) and recorded at 25 Hz frame collection rate by a Super VHS (Panasonic SVHS AG-5700). The CCD images were digitised from tape and processed for presentation. The result is shown in figure 6. The cell on the top in figure 6 A, which a brightfield image of two progenitor cells in close contact with each other, was electroporated in the presence of fluorescein as described above. As shown in the fluorescence image in figure 6 B, the fluorescence is uniquely localised to the upper cell with virtually zero spillover of fluorescein to the neighbouring cell. Thus, with the current electrodes and instrumentation, it is feasible to achieve a sufficient spatial resolution to electroporate single cells.

Example 2. Electroporation of intracellular organelles in progenitor cells from adult rat brain using extracellular electrodes

The experimental set-up from example 1 was repeated for *in situ* electroporation of organelles in single progenitor cells. Specifically, fluorescein was introduced into single cells by ten 0.5-Hz superthreshold field applications resulting in potential shifts at the cell membrane of $1.6 \text{ V} \pm 0.07 \text{ V}$ (range 1.5-2.4 V), and compared to cells electroporated at low membrane potentials of 0.5-1.0 V. Following electroporation, the extracellular dye-containing media was replaced by a Hank Hepes solution. The result of electroporation at high voltages is shown in figure 7 A, where punctuate fluorescence is observed in the exo-nuclear cytoplasmic region, but not in the nuclear region which contains a smaller number of organelles. In total, eighteen out of twenty cells electroporated in this protocol displayed a similar staining pattern, reminiscent of what is observed using an organelle-specific dye such as rhodamine 123, which labels the mitochondrial fraction of organelles. In comparison, cells electroporated at the plasma membrane threshold po-

tential, generally displayed a diffuse fluorescence from fluorescein entrapped in the cytosol, as shown in figure 7 B. This observation of a differential localisation of fluorescein comparing application of low and high electric field strengths is consistent with the higher breakdown potential of intracellular organelles as predicted by equation 1 given in the background part. owing to their smaller dimension. It has been shown that 2.5-10 fold higher electric field strengths are required for electroporation and fusion of isolated mitochondria compared to 10-mm diameter cells. The results are also in accord with morphological observations in giant squid axons following mild, moderate, and severe electroporation protocols.

CLAIMS

1. A method for permeabilisation of a cell structure consisting of a single cell, an intracellular structure or an organelle, characterised in that it comprises the following steps:

- 5 (a) microelectrodes are provided;
(b) the microelectrodes are connected to a power supply;
(c) the electrodes, individually controlled by high-graduation micromanipulators, are placed close to the
10 cell structure at an appropriate inter-electrode distance;
(d) a highly focused electric field of a strength sufficient to obtain electroporation is applied between the electrodes.

15 2. A method according to claim 1, wherein said cell structure is an intracellular structure or intracellular organelle, and said microelectrodes are arranged so that the ends of the electrodes are placed within the cell containing the cell structure.

20 3. A method according to claim 1 or 2, wherein two electrodes are used.

4. A method according to any one of the claims 1 - 3, wherein the power supply generates a voltage at the membrane of the cell structure from 10 mV to 100 V.

25 5. A method according to any one of the claims 1 - 4, wherein the inter-electrode distance is less than 10 mm.

6. A method according to claim 5, wherein said distance is less than 100 μ m.

30 7. A method according to any one of claims 1 - 6, wherein the diameter of the ends of the electrodes closest to the cell structure is from a few nanometres to a few micrometres.

8. A method according to any one of claims 1 - 7, wherein the electric field applied in step (b) is obtained by a rectangular DC voltage pulse.

5 9. A method according to claim 8, wherein the length of the pulse is from 0,1 μ s to several minutes.

10 10. A method according to any one of claims 1 - 9, wherein the electrodes are positioned by use of a microscope, at least one micropositioner, and/or a stereotactic device.

11. A method according to any one of claims 1 - 10; wherein cell-impermeant solutes are transported through the pores created in the cell structure by the electroporation.

12. A method according to claim 11, wherein the solutes before the electroporation are comprised in an extracellular medium.

13. A method according to claim 11, wherein the solutes before the electroporation are comprised in a medium entrapped in the cell structure.

20 14. A method according to claim 13, wherein the entrapped solutes comprise a pharmaceutically active compound.

15. A method according to any one of claims 11 - 14, wherein the medium used to dissolve the solutes is a physiological buffer.

25 16. A method according to any one of the claims 11 - 15, wherein the medium comprising the solutes is delivered to the cell structure by use of a catheter.

17. A method according to any one of claims 1 - 16, wherein at least one electrodes is a carbon fibre electrodes.

18. A method according to any one of the claims 1 - 16, wherein at least one electrode is a hollow electrode.

35 19. A method according to claim 18, wherein the current needed for electroporation is carried by an intra-electrode electrolyte.

20. A method according to claim 17 or 18, wherein the medium comprising the solutes is delivered to the cell structure through the hollow fibre electrode.

21. A method according to claim 20, wherein the solutes are delivered to the cell structure by electrophoresis or electroosmosis.

22. A method according to any one of the claims 18 - 21, wherein said hollow electrodes are fused silica electrodes.

23. A method according to any one of claims 1 - 22, wherein the cell structure is a liposome.

24. Use of the method according to any one of claims 1 - 23 for administration of drugs.

25. Use of the method according to any one of claims 1 - 23 for administration of genes.

26. Use of the method according to any one of claims 1 - 23 for administration of biochemical markers and dyes.

27. Use of the method according to any one of claims 1 - 23 for administration of nanoparticles.

28. Use of the method according to any one of claims 1 - 23 in a biosensor application.

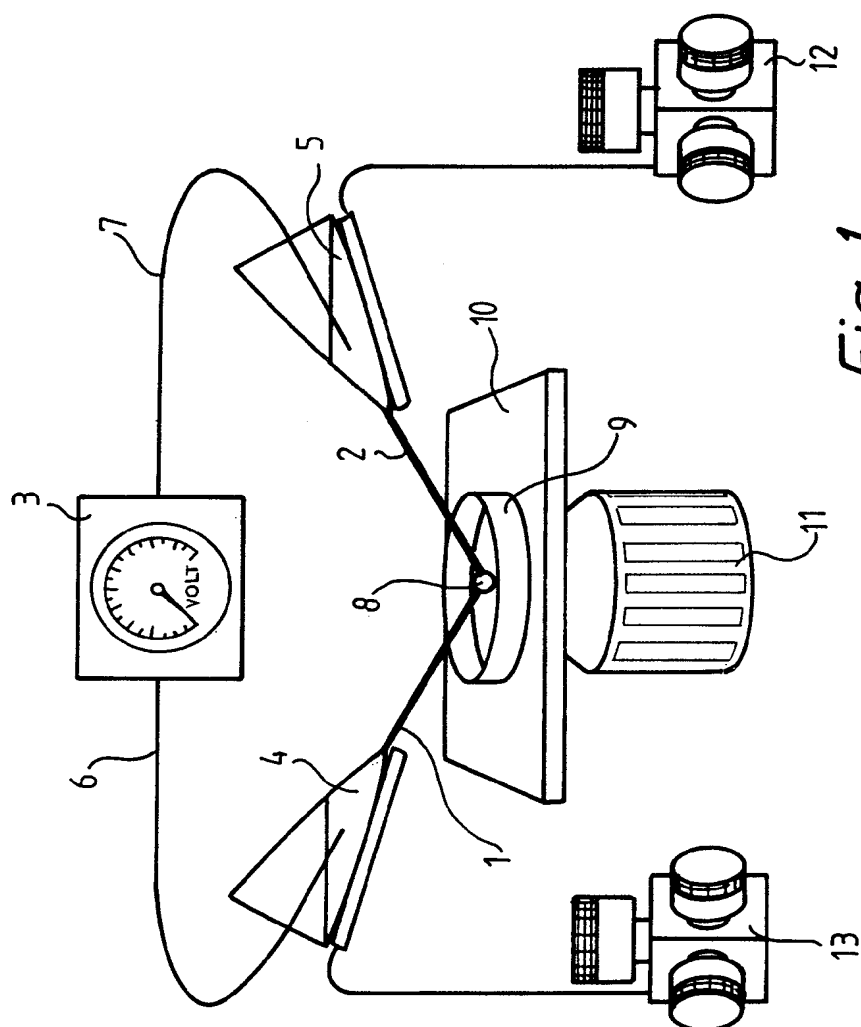
29. Use according to claim 28, in combination with a chemical separation.

30. Use of the method according to any one of claims 1 - 23 in treatment of tumours.

31. Use of the method according to any one of claims 1 - 23 in treatment of chronic neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease.

32. Use of the method according to any one of claims 1 - 23 for the study of cellular migration, proliferation, growth, differentiation biochemistry and/or biophysics.

1/7



2/7

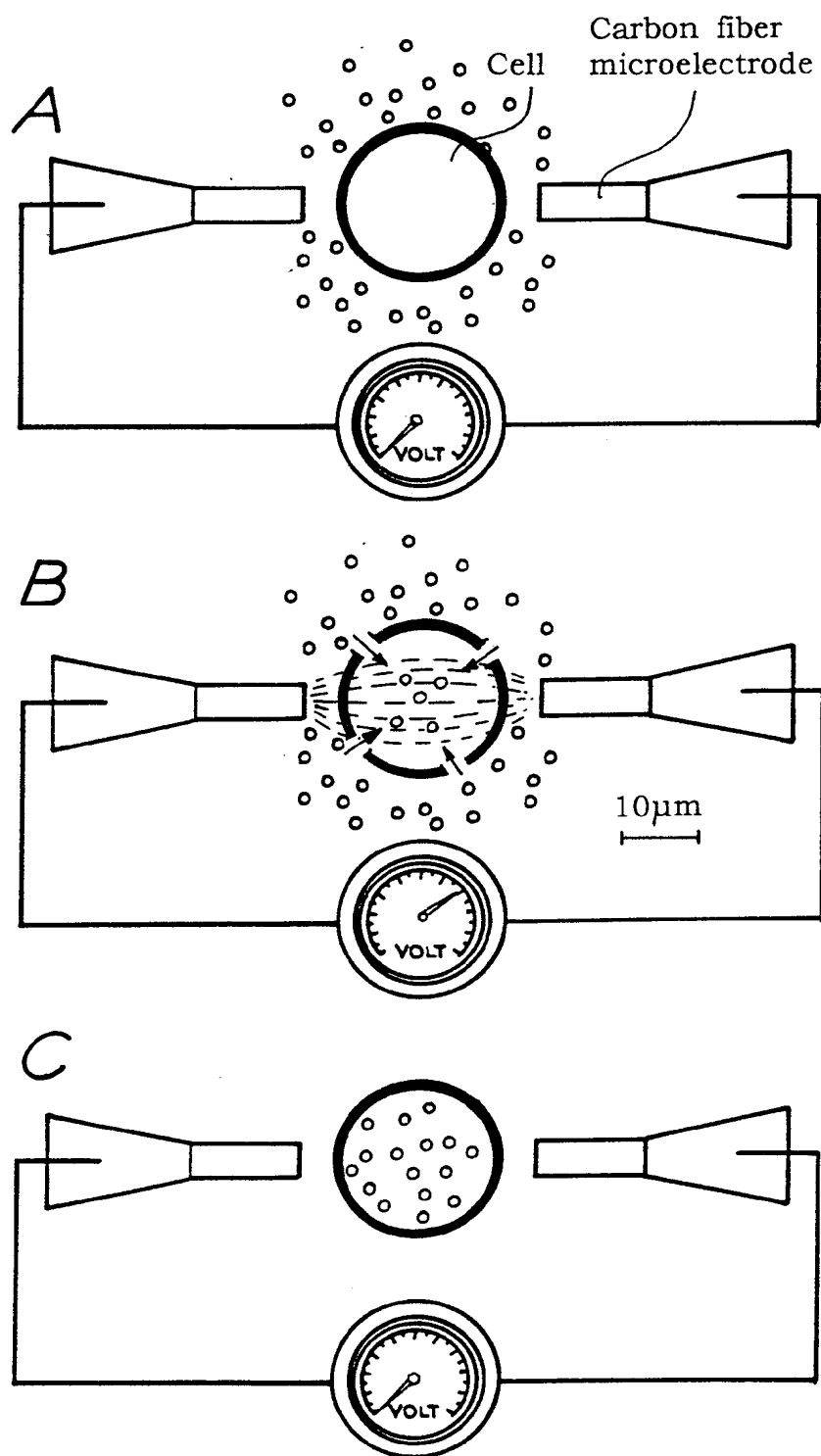
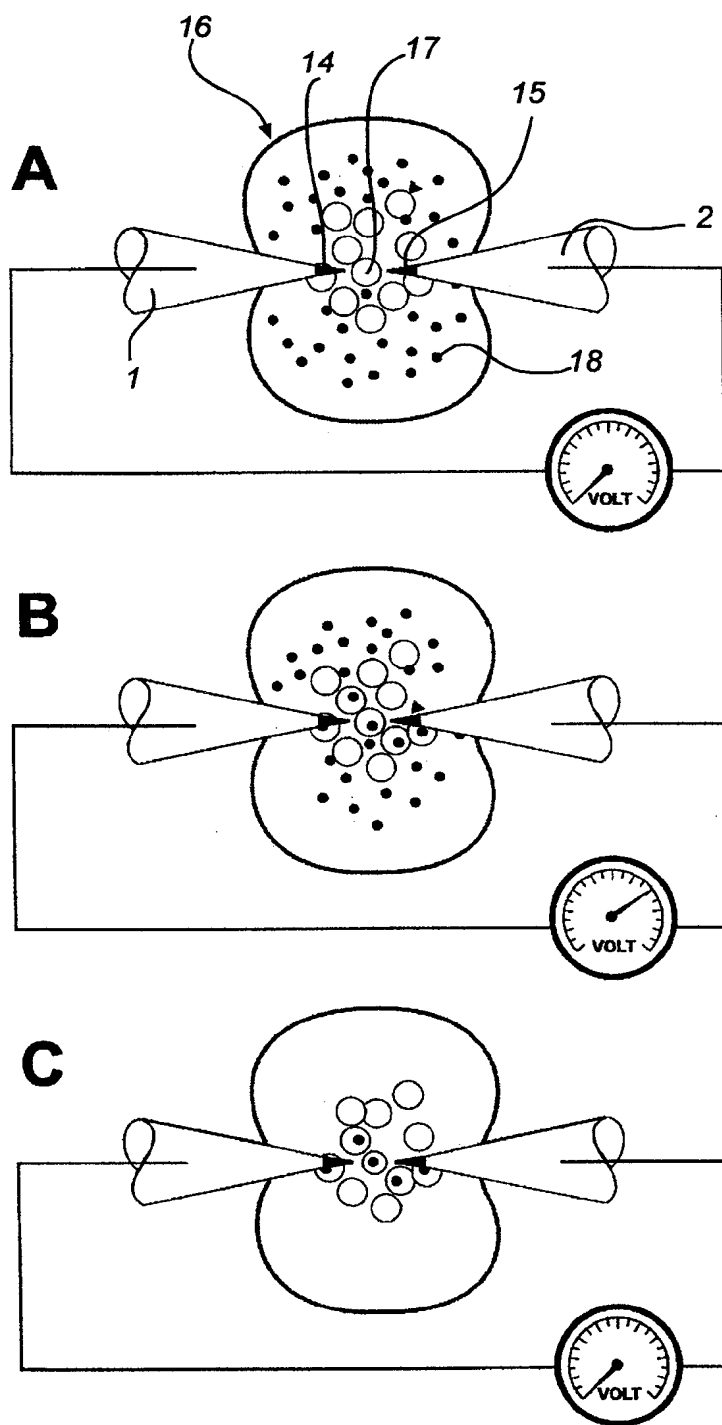


Fig. 2

3/7



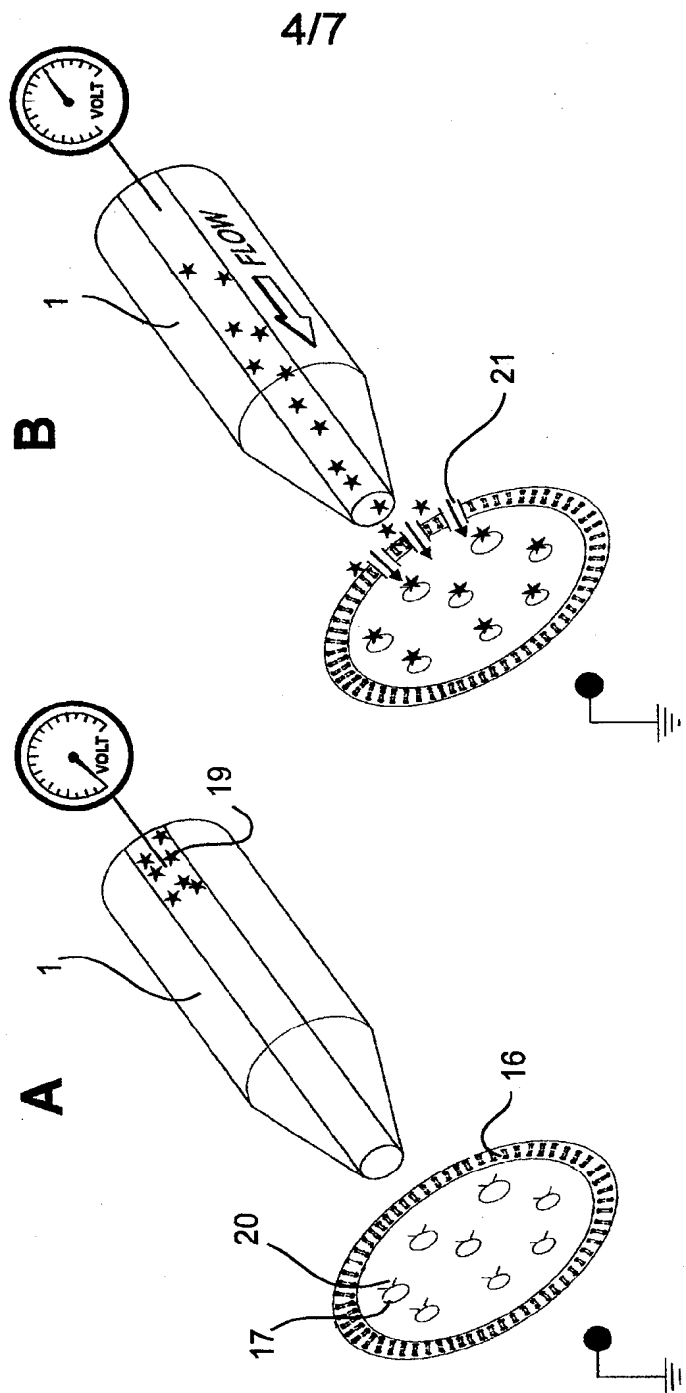
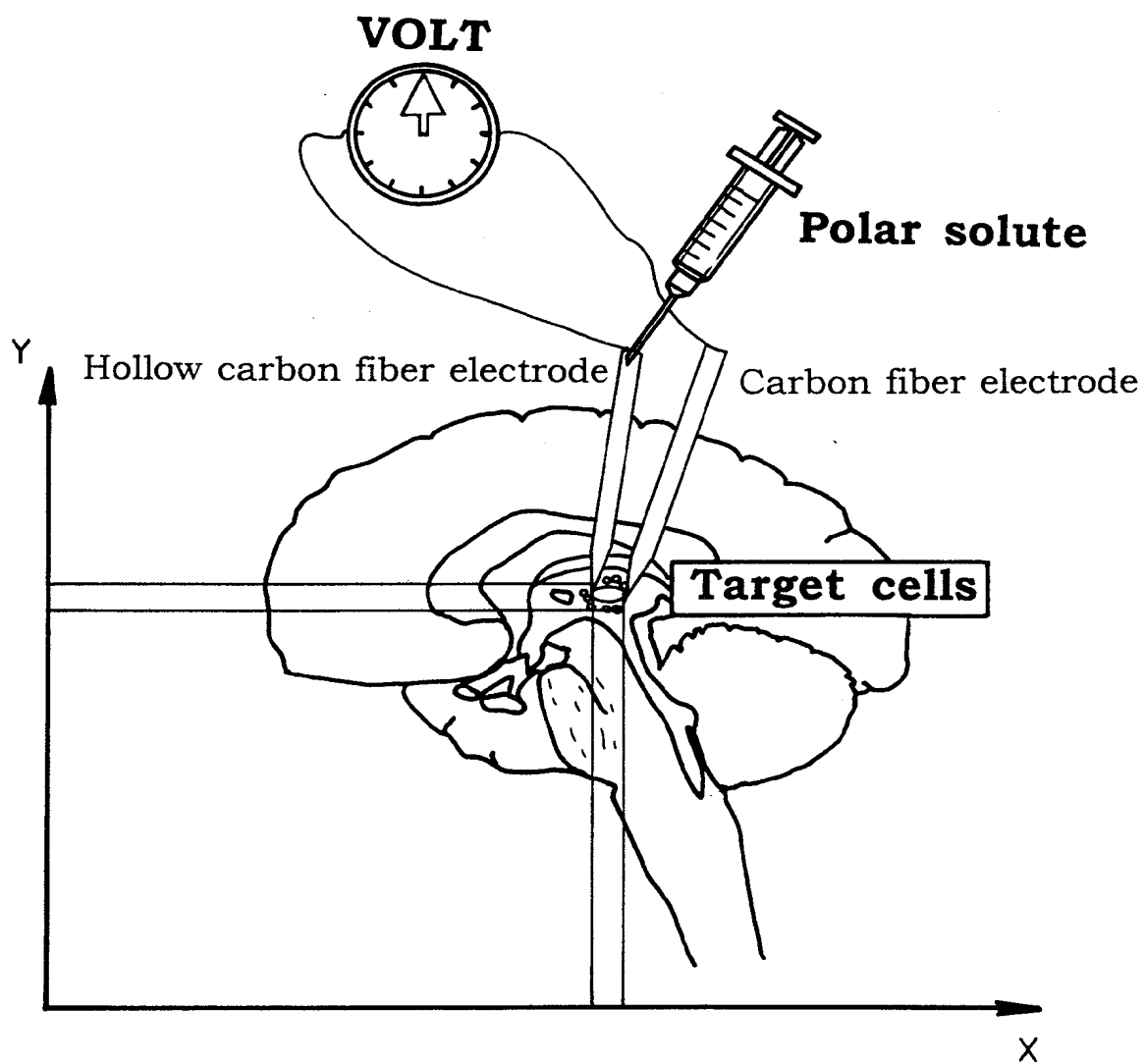


Fig. 4

5/7

*Fig. 5*

6/7

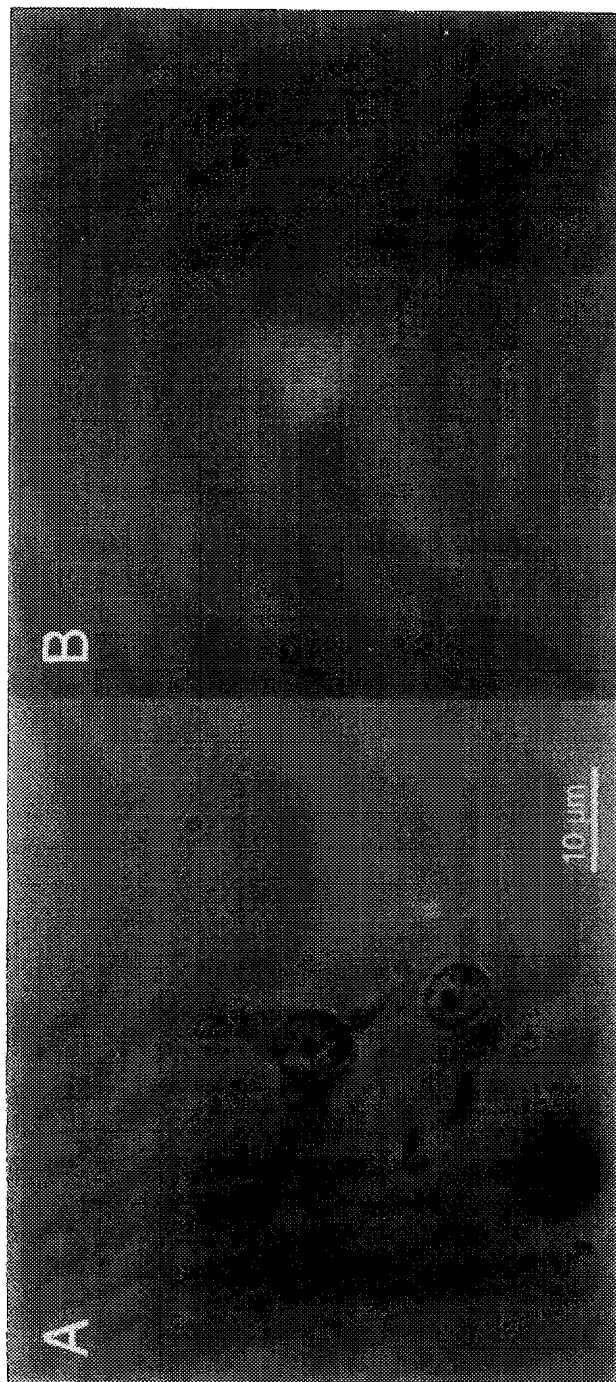


Fig. 6

7/7

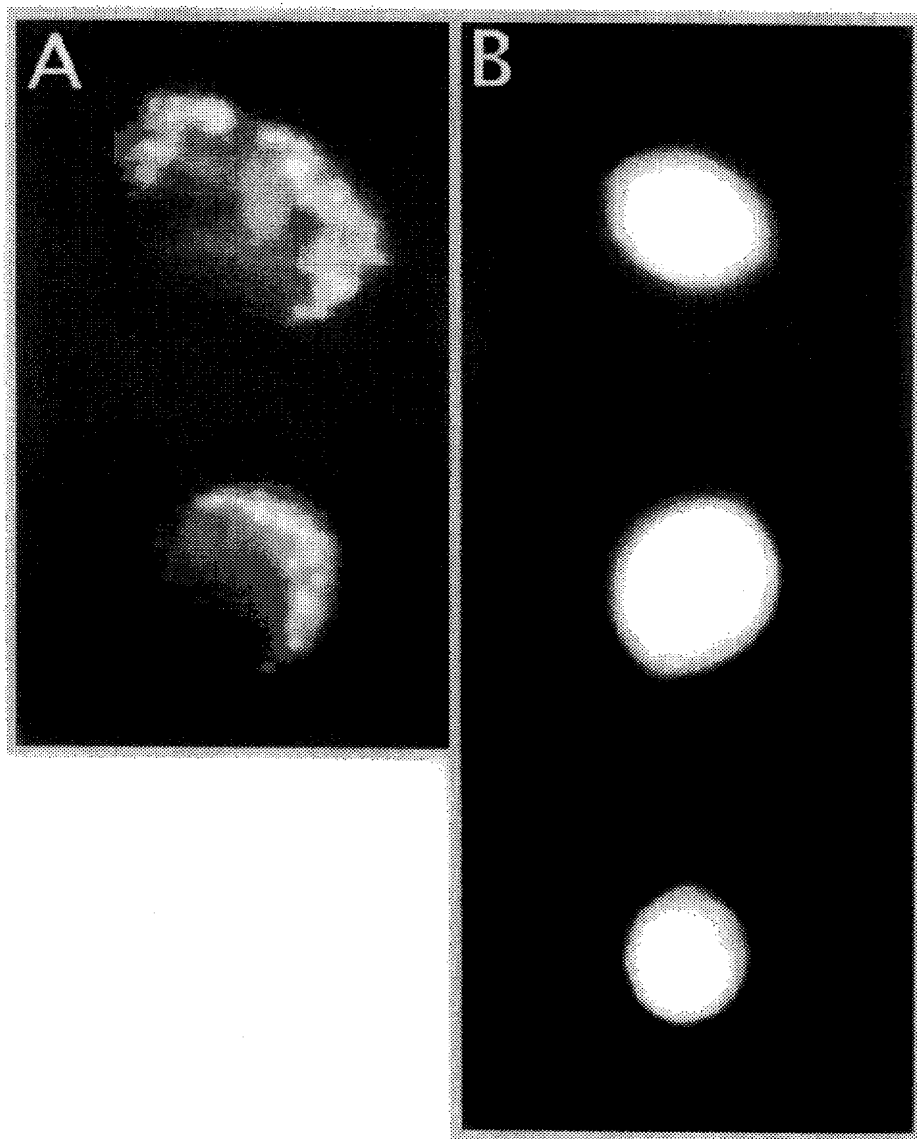


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/02012

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61N 1/32, C12N 13/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61N, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, SCISEARCH, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Proc. Natl. Acad. Sci., Volume 95, Sept 1998, J. Anders Lundqvist et al, "Altering the biochemical state of individual cultured cells and organelles with ultramicroelectrodes", page 10356 - page 10360 --	1-32
A	Biophysical Journal, Volume 73, Sept 1997, P.E. Marszalek et al, "Kinetics of Release of Serotonin from Isolated Secretory Granules. I. Amperometric Detection of Serotonin from Electroporated Granules", page 1160 - page 1168, See abstract, fig 1, materials and methods --	1-32

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 February 1999

Date of mailing of the international search report

27 -02- 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/02012

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9639226 A1 (GENETRONICS, INC.), 12 December 1996 (12.12.96), Abstract, claims, figures -- -----	1-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/02012

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 30-31
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

